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APPLICATION NUMBER: ***60/349,929***
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PATENT

Attorney Docket No.: NIH-05102

PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

Docket Number	NIH-05102	Type a plus sign (+) inside this box →	

INVENTOR(s) / APPLICANT(s)			
Last Name	First Name	Middle Initial	Residence (City and Either State or Foreign Country)
Pavan	**William**	**J.**	**Derwood, Maryland**
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TITLE OF THE INVENTION (280 Characters Max.)

Alteration of RAB38 Function To Modulate Mammalian Pigmentation

CORRESPONDENCE ADDRESS

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ENCLOSED APPLICATION PARTS *(Check All That Apply)*

X	Specification	Number of Pages	41	X	Other (Specify): Power of Attorney
X	Drawing(s)	*Number of Sheets*	7	X	Other (Specify): Assignment
				X	Other (Specify): Sequence Listing

METHOD OF PAYMENT OF FILING FEES FOR THIS PROVISIONAL APPLICATION FOR PATENT

X	The filing fee for this provisional application in the amount of **$160.00** is enclosed.	FILING FEE AMOUNT ($)	**$160.00**
X	The Commissioner is hereby authorized to charge any deficiency in the payment of the required fee(s) and/or credit any overpayment to Deposit Account No.: **08-1290. An originally executed duplicate of this transmittal is enclosed for this purpose.**		

This invention was made in part with support by an agency of the United States Government.

___ No.
X Yes, the name of the U.S. Government agency is: National Institutes of Health.

Respectfully submitted,

Date: January 18, 2002

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___ Additional inventors are being named on separately numbered sheets attached hereto.

Express Mail Label No.: [redacted]08 738 914 US

PATENT
Attorney Docket No.: NIH-05102

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: William J. Pavan et al.
For: **Alteration of RAB38 Function To Modulate Mammalian Pigmentation**

Box Provisional Patent Application
Assistant Commissioner for Patents
Washington, D.C. 20231

CERTIFICATION UNDER 37 C.F.R. § 1.10

I hereby certify that this correspondence and the documents referred to as attached therein are being deposited with the United States Postal Service on **January 18, 2002**, in an envelope as "EXPRESS MAIL POST OFFICE TO ADDRESSEE" service under 37 C.F.R. § 1.10, Mailing Label Number **EV 008 738 914 US** addressed to: **Box Provisional Patent Application,** Assistant Commissioner for Patents, Washington, D.C. 20231.

James R. Davenport

TRANSMITTAL COVER SHEET FOR FILING PROVISIONAL APPLICATION (37 C.F.R. § 1.51(2)(i))

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 C.F.R. 1.53(b)(2).

1. The following comprises the information required by 37 C.F.R. § 1.51(a)(2)(i)(A):

2. The name(s) of the inventor(s) is/are (37 C.F.R. § 1.51(a)(2)(i)(B)):

   William J. Pavan
   Stacie K. Loftus

3. Address(es) of the inventor(s), as numbered above (37 C.F.R. § 1.51(a)(2)(i)(C)):

   7724 Warbler Lane, Derwood, Maryland 20855
   10829 Monticello Drive, Great Falls, Virginia 22066

4. The title of the invention is (37 C.F.R. § 1.51(a)(2)(i)(D)):

   Alteration of RAB38 Function To Modulate Mammalian Pigmentation

5. The name, registration, and telephone number of the attorney (*if applicable*) is (37 C.F.R. § 1.51(a)(2)(i)(E)):

Kamrin T. MacKnight
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(complete the following, if applicable)

X A Power of Attorney accompanies this cover sheet.

6. The docket number used to identify this application is (37 C.F.R. § 1.51(a)(2)(i)(F)):

Docket No.: **NIH-05102**

7. The correspondence address for this application is (37 C.F.R. § 1.51(a)(2)(i)(G)):

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8. Statement as to whether invention was made by an agency of the U.S. Government or under contract with an agency of the U.S. Government. (37 C.F.R. § 1.51(a)(2)(i)(H)):

This invention was made in part with support by an agency of the United States Government.

___ No.

X Yes.

The name of the U.S. Government agency is:

National Institutes of Health

9. Identification of documents accompanying this cover sheet:

A. Documents required by 37 C.F.R. § 1.51(a)(2)(ii)-(iii):

Specification: No. of pages **41**

Drawings: No. of sheets **7**

B. Additional documents:

X Claims: No. of claims **41**

X Power of Attorney

X Assignment

X Sequence Listing

10. Fee

The filing fee for this provisional application, as set in 37 C.F.R. § 1.16(k), is $160.00, for other than a small entity, and $80.00, for a small entity.

___ Applicant is a small entity.

11. Fee payment being made at this time

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Date: January 18, 2002

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ALTERATION OF RAB38 FUNCTION TO MODULATE MAMMALIAN PIGMENTATION

FIELD OF THE INVENTION

The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

BACKGROUND OF THE INVENTION

Melanocytes are specialized pigment producing cells that are responsible for coloration of skin, eyes and hair. Coat color alterations resulting from melanocyte defects are easily identifiable in mice. These mouse mutants are proving valuable for the identification of candidate human disease genes and for the elucidation of mechanisms underlying cellular function. To date, there are approximately 100 loci in the mouse that, when mutated, affect pigmentation. However, the underlying genetic defect has not been identified in about 60 of these loci (*See,* The Jackson Laboratory's Mouse Genome Informatics web site).

Disorders with reduced pigmentation can be placed into two groups according to whether they affect melanocyte differentiation or whether they affect the function of the pigment producing organelle in the melanocyte, the melanosome. Examples of the first group include Piebaldism and Waardenburg Syndrome, characterized by a localized absence of melanocytes resulting in "white patch" patterns. Genes affected in these disorders, *KIT, MITF, PAX3, SOX10, EDNRB, EDN3*, are involved in specification, migration and survival of the melanocyte lineage (Jackson, Hum Mol Genet 6:1613-1624 [1997]). Mouse models of these disorders have characteristic spotted coat patterns (Jackson, Hum Mol Genet 6:1613-1624 [1997]). Oculocutaneous albinism (OCA) I-IV, Chediak-Higashi Syndrome (CHS), Hermansky-Pudlak Syndrome (HPS) I-III and Griscelli syndrome (GS) correspond to the second group.

The molecular defects contributing to the reduced pigmentation in OCA occur in genes (*TYR*, *TYRP1*, *P* and *AIM1)* that mainly effect melanosome formation and the amount and type of melanin pigment formed (King *et al.*, in Scriver *et al.*, (eds.) The metabolic basis of inherited disease, 7th ed. (McGraw-Hill, New York) pp. 4353-4392 [1995]); and Newton *et al.*, Am J Hum Genet 69:981-988 [2001]). Genes responsible for HPS, CHS and GS are involved in the regulation of vesicle traffic including melanosomes within the cell and include *HPS1*, *AP3*, *HPS3*, *CHS1*; *MYO5A* and *RAB27A* (Jackson, Hum Mol Genet 6:1613-1624 [1997]; and Marks and Seabra, Nat Rev Mol Cell Biol 2:738-748 [2001]).

Although many genes have been associated with pigmentation disorders, in view of the genetic heterogeneity of these disorders in both mice and humans, there is a need in the art to identify additional candidate disease genes in these and other species.

SUMMARY OF THE INVENTION

The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

The present invention provides an isolated nucleic acid that comprises a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12. In some embodiments, the nucleic acid is deoxyribonucleic acid. In other embodiments, the nucleic acid is the complement of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12. In related embodiments, a vector comprising the nucleic acid is provided. Also provided is a host cell comprising the vector. Additionally, the present invention provides a protein encoded by the nucleic acid sequence set forth in SEQ ID NO:9.

In some embodiments, the present invention provides methods for detecting mutations in *Rab38* comprising the steps of: amplifying at least a portion of *Rab38* from genomic DNA to yield a *Rab38* amplifcation product; purifying the *Rab38* amplification product; and sequencing the *Rab38* amplification product. In preferred embodiments, the amplifying is accomplished using a polymerase chain reaction. In related embodiments, the at least a portion of *Rab38* genomic DNA is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence. In some particularly preferred embodiments, the at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3. In some embodiments, the genomic DNA is mammalian genomic DNA. Also provided are embodiments where the purifying is accomplished via size selection.

The present invention further provides methods for detecting mutations in *Rab38* comprising the steps of: amplifying at least a portion of *Rab38* from genomic DNA to yield a *Rab38* amplification product; digesting the *Rab38* amplification product to yield a digested *Rab38* amplification product; and electrophoresing the digested *Rab38* amplification product. In preferred embodiments, the amplifying is accomplished using a polymerase chain reaction. In some embodiments, the at least a portion of *Rab38* genomic DNA is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence. In preferred embodiments, the at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3. In particularly preferred embodiments, the genomic DNA is mammalian genomic DNA.

The present invention also provides methods for screening for biologically active agents to modulate RAB38 activity, comprising the steps of: providing: melanocytes comprising RAB38 activity, and a candidate agent; and exposing the melanocytes to the candidate agent to yield treated melanocytes; and measuring the modulation of the RAB38 activity of the treated melanocytes by the candidate agent.

In some embodiments, the RAB38 activity comprises GTPase activity. In some related embodiments, the RAB38 activity comprises GTP binding activity or GDP release. In some preferred embodiments, the RAB38 activity comprises TYRP1 trafficking to melanosomes or RAB38 trafficking to melanosomes.

In some embodiments, the present invention provides kits for screening for biologically active agents that modulate RAB38 activity, comprising: plurality of melanocytes comprising RAB38 activity, wherein the melanocytes are provided within a container, and instructions for determination of RAB38 activity in the melanocytes. In some preferred embodiments, kits further comprise the means to analyze RAB38 activity. In some related embodiments, the means to analyze RAB38 activity comprises an assay to assess GTPase activity, an assay to assess GTP binding activity, an assay to assess GDP release, an assay to assess TYRP1 trafficking to melanosomes, or an assay to assess RAB38 trafficking to melanosomes.

The present invention also provides kits for the detection of mutations in *RAB38* comprising at least two primer sequences suitable for amplification of at least a portion of *RAB38*, and instructions for utilizing the kit. In some preferred embodiments, the primer sequences are selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22. In some related embodiments, the kit is suitable for use in the polymerase chain reaction. The invention also provides embodiments further comprising reagents for digesting nucleic acid.

In some embodiments, the present invention also provides kits for diagnosing defects in melanosome function, comprising melanocytes comprising *RAB38* and instructions for assessing defects in melanosome function. In some preferred embodiments, the kits further comprise the means to analyze RAB38 activity. In some related embodiments, the means to analyze RAB38 activity comprises an assay to assess GTPase activity, an assay to assess GTP binding activity, an assay to assess GDP release, an assay to assess TYRP1 trafficking to melanosomes or an assay to assess RAB38 trafficking to melanosomes.

DESCRIPTION OF THE FIGURES

Figure 1 depicts a microarray expression profile of various pigmentation control genes and *Rab38*. Cluster analysis identified nine genes known to be involved in pigmentation. Columns to the right list mouse and human pigmentation disorders corresponding to a given gene. *Rab38* (arrow) was found within the same expression profile cluster as the nine control genes known to be involved in pigmentation. Hybridized sample cell lines are listed across the top of the expression profile. Relative gene expression is evaluated as a calibrated ratio (sample cell line / MnSOD6cl1 reference). Pseudocolor scale for ratio values is shown below.

Figure 2 illustrates that RAB38, like known melanogenic enzymes, is expressed in the retinal pigmented epithelium (RPE). All panels show the eye at embryonic day E11.5. The melanogenic enzymes expressed in the RPE at this developmental stage include tyrosinase (*Tyr*; Panel A), tyrosinase related protein 1 (*Tyrp1*; Panel B), and dopachrome tautamerase (*DCT/Tyrp2*; Panel C). Additional control genes from the microarray cluster data that are expressed in the RPE include melastatin1 (*Mlsn*; Panel D) and *Aim1/Matp* (Panel E). *Rab38* is expressed in the RPE at E11.5 (Panel F) and at E10.5 and E12.5. The scale bar equals 100 μm.

Figure 3 illustrates the *Rab38* map location and the phenotype of the chocolate (*cht/cht*) mouse. Panel A provides a map of human chromosome 11 showing that human *Rab38* maps 1.4 Mb distal to tyrosinase and 2.5 cM proximal to EED genes. In the corresponding mouse chromosome 7, syntenic region gene order is conserved (*e.g.*, the *cht* locus maps to the same interval as *Rab38*). Panel B provides a photograph of C57Bl/6J +/+ (left side, black) and C57Bl/6J $Rab38^{cht}/Rab38^{cht}$ (right side, brown) mice. Panel C shows the eyes from 2 day old wildtype C57Bl6/J +/+ mice with normal pigmentation, while Panel D shows that the eyes from $Rab38^{cht}/Rab38^{cht}$ mice exhibit much less pigmentation.

Figure 4 indicates that the *Rab38* mutation causes the *chocolate* (*cht*) mouse phenotype. Panel A provides a comparison of *Rab38* sequence between wildtype C57Bl6/J +/+ DNA (SEQ ID NO:1) and mutant C57Bl/6J $Rab38^{cht}$/+ DNA (SEQ ID

NO:2), revealing a G146T nucleotide change (arrow) in the *cht* allele. This nucleotide change was never seen in eight additional inbred strains analyzed. Panel B illustrates that the G146T mutation creates a *Sex*A1 restriction enzyme site in C57Bl/6J *Rab38^{cht}/Rab38^{cht}* DNA and ablates a *Bsa*JI restriction site present in wildtype *Rab38* sequence. A 216 bp region surrounding the G146T nucleotide mutation was amplified from both C57Bl/6J +/+ DNA and C57Bl6/J *Rab38^{cht}/Rab38^{cht}* DNA. *Sex*A1 digests the PCR fragment of C57Bl/6J *Rab38^{cht}/Rab38^{cht}* (lane 1), but not C57Bl/6J +/+(lane 2); *Bsa*J1 digests the PCR fragment of C57Bl6/J +/+ (lane 4), but not C57Bl/6J *Rab38^{cht}/Rab38^{cht}*(lane 3).

Figure 5 shows that RAB38 G19 is located in the GTP binding pocket. Panel A provides the 3-dimensional location of the amino acid G19 of RAB38 in relation to the nucleotide binding site, as determined using the molecular modeling database (MMDB; Wang *et al.*, Nucleic Acids Res 28:243-245 [2000]), based upon the crystal structure for RAB3a (MMDB 10125; and Dumas *et al.*, Structure Fold Des 7:413-423 [1999]). Overlaying the RAB38 sequence with that of RAB3a identified amino acid S32 of RAB3a as being equivalent to G19 of RAB38. The program Cn3D 3.0 was used to indicate the location of the RAB38 G19 (white), predicting interaction with the bound nucleotide. Protein structure is indicated by color: green, α- helices; gold, β sheet; blue, random coils; white, site of RAB3a S32 equivalent to RAB38 G19 located at the nucleotide binding site; grey, Mg^{++} ion; red-grey, GppNHp nucleotide analog. Panel B provides alignments of the highly conserved N-terminal region including human RAB38 (NP_071732; and SEQ ID NO:3), rat RAB38 (AAA42000; and SEQ ID NO:4), and mouse RAB38 (AK009296.1; and SEQ ID NO:5) amino acid sequences; and human RAB3a (P20336; and SEQ ID NO:6), human RAB5 (F34323; and SEQ ID NO:7) and human N-RAS (TVHURA; and SEQ ID NO:8) amino acid sequences. Sequence alignment was done using the ClustalW algorithm (Smith *et al.*, Genome Res 6:454-462 [1996]). Bars indicate highly conserved regions that occupy the nucleotide binding pocket, observed in the X-ray crystal structure of RAB3a (Ostermeier and Brunger, Cell 96:363-374 [1999]). Black denotes sequence identity,

grey denotes sequence conservation, and red denotes the conserved amino acid that is mutated in the chocolate mice.

Figure 6 illustrates that *cht/cht* melanosomes are similar in morphology to *Tyrp1*^{b} melanosomes. Bright field images of melanosomes are shown from the periphery of primary cultured melanocytes, isolated from C57Bl6/J +/+ mice in Panel A, and from C57Bl/6J *Rab38*^{cht}/*Rab38*^{cht} mice in Panel B. Melanosomes from wildtype melanocytes are oval and darkly pigmented, while those from C57Bl/6J *Rab38*^{cht}/*Rab38*^{cht} melanocytes are smaller, more circular and less pigmented, resembling melanosomes from *Tyrp1*^{b}/*Tyrp1*^{b}, melan-b cells. The scale bar equals 2 μm.

Figure 7 shows that RAB38 is a melanosomal protein needed for appropriate TYRP1 trafficking. Bright field and matching confocal images of identical exposure of melanosomes in the periphery of primary melanocytes cultures are provided in the upper and lower panels respectively. Panels A and B provide images of C57Bl/6J +/+ melanosomes, Panels C and D provide images of C57Bl/6J *Rab38*^{cht}/*Rab38*^{cht} melanosomes, and Panels E and F provide images of melanosomes from melan-a cells transfected with a GFP-RAB38 expression construct. TYRP1 distribution was revealed by MEL5 staining in Panels B and D, while GFP-RAB38 immunofluorescence shown in Panel F, demonstrates co-localization of the GFP-RAB38 signal with the highly pigmented, end stage melanosomes. The scale bar for Panels A and D equals 1.6 μM, while that for Panels E and F equals 2.4 μM.

DESCRIPTION OF THE INVENTION

The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

In order to meet a need in the art for identification of additional pigmentation disease genes, expression profile analysis using cDNA microarrays was conducted done to develop the present invention. Expression profile analysis was utilized for the development of the present invention, as it is a powerful tool for organizing the common patterns found among thousands of gene expression measurements, and for identifying genes with similar distinctive expression patterns among multiple experimental samples (Eisen *et al.*, Proc Natl Acad Sci USA 95:14863-14868 [1998]). Analysis of genes contained within a cluster has revealed that these genes are often functionally related within the cell (Eisen and Brown, Methods Enzymol 303:179-205 [1999]; and Mody *et al.*, Proc Natl Acad Sci USA 98:8862-8867 [2001]). As detailed below, by using this approach *Rab38* was identified as a candidate pigmentation gene. Further analysis confirmed that RAB38 is a melanosomal protein, mutated in the mouse pigmentation mutant, *chocolate* (*cht*), and important for the sorting of the melanosomal protein TYRP1 in melanocytes. These experiments conducted during the development of the present invention provide the first successful use of microarray expression profiling to identify a mammalian pigmentation disease gene.

Expression Profile Analysis Identifies *RAB38* as a *chocolate* Candidate Gene

In order to identify novel and uncharacterized genes involved in melanocyte function and disease, a collection of cDNA clones to be used for expression profile and functional analyses were generated (Loftus *et al.*, Proc Natl Acad Sci USA 96:9277-9280 [1999]). cDNA clones from IMAGE consortium library 2NbHM (*See*, The National Center for Biotechnology Information's web site for UniGene Library No. 198) were previously shown to be appropriate for gene expression studies aimed at understanding melanocyte development and function (Loftus *et al.*, Proc Natl Acad Sci USA 96:9277-9280 [1999]; and Loftus and Pavan, Pigment Cell Res 13:141-146 [2000]). For this analysis, 4356 cDNA clones from library 2NbHM were printed to glass slides. A total of 17 cell lines representing neural crest and non-neural crest derived tissues were used in this analysis. Included were eleven melanoma cell lines

(Bittner *et al.*, Nature 406:536-540 [2000]), three rhabdomyosarcoma cell lines, one glioblastoma cell line, HeLa cells, and 293T cells. Array hybridizations for each of these cell lines were performed in a pair-wise fashion, using RNA from cell line MnSOD6 cl1 as a reference. MnSOD6 cl1 is an amelanotic melanoma cell line rendered non-tumorigenic by the introduction of a region of human chromosome 6 (Trent *et al*, Science 247:568-571 [1990]). MnSOD6 cl1 has been used previously for expression profile analysis of melanoma lines using a different set of cDNA clones (Bittner *et al.*, Nature 406:536-540 [2000]).

Hierarchical cluster analysis found nine genes (*DCT, TYRP1, PMEL17, AIM-1, MELAN-A/MART1, MLSN, ATRN, PAX3* and *CHS1*), previously known to be involved in melanocyte function, to cluster together (*See,* Figure 1). On closer analysis of the hierarchical clustering data an additional gene, *Rab38*, was found to have a similar expression variation to these melanocyte genes (*See,* Figure 1). Four of the nine melanocyte genes examined (*TYRP1, DCT, MLSN* and *AIM1*) were expressed in the melanocytes of the retinal pigment epithelium (RPE) at E11.5 (*See,* Figure 2). Consistent with the placement of *Rab38* within this cluster of genes, whole mount *in situ* analysis demonstrated that *Rab38* was also expressed in the melanocytes of the RPE at this age (*See,* Figure 2). Northern blot analyses revealed that *Rab38* expression was restricted to the melanocyte derived cell lines (Jager *et al.*, Cancer Res 60:3584-3591 [2000]).

Utilizing the recently available human genome sequence, *Rab38* was determined to be located on human chromosome 11, flanked proximally by *TYR* and distally by *EED* and *MYO7A* (*See,* Figure 3, Panel A). A conserved linkage group on mouse chromosome 7 was identified by comparison of the human genome map with the mouse genome mapping data (*See,* The Jackson Laboratory's web site for Mammalian Homology Query). Closer analysis of loci in the mouse conserved linkage group indicated that an uncloned mouse pigmentation mutant, *cht*, was contained within this interval (*See,* Figure 3, Panel A; and Potter and Rinchik, Mamm Genome 4:46-48 [1993]). The *cht* mutation arose as a spontaneous, isogenic mutation on an

inbred C57Bl/6J background and has been maintained on this background since 1984 (Macpike and Mobraaten, Mouse News Lett 700:86 [1984]). *Cht/cht* mice are identifiable at birth by lighter skin and eyes, and at weaning by a deep-brown coat color when compared to the C57Bl/6J parental strain (*See,* Figure 3, Panels B and C). Thus, *Rab38* was implicated as a candidate gene for the *cht* locus based on genomic map positions and the expression of *Rab38* in cell types affected in *cht/cht* mice, RPE and melanocyte derivatives.

Mutation of *Rab38* Causes Melanocyte Defects in *cht/cht* Mice

Genomic sequence flanking exon/intron boundaries for the three mouse *Rab38* exons was obtained from mouse trace archive genomic sequence (*See,* National Center for Biotechnology Information web site for Trace Archive Querying). DNA from C57Bl/6J *cht/+* animals was obtained from Jackson Laboratories Mouse DNA Resource, amplified using genomic *Rab38* primers and directly sequenced. In exon 1, a G146T nucleotide mutation was identified in the *cht* allele (*See,* Figure 4, Panel A). This sequence alteration was confirmed by restriction digest in multiple *cht/cht* DNA samples, as the resulting nucleotide substitution changes a *BsaJI* site (CCNNGG) to a *Sex*A1 restriction site (ACCWGGT) (*See,* Figure 4, Panel B). This sequence alteration was not detected in analysis of 8 additional inbred mouse strains (CAST/Ei, SPRET/Ei, 129/SVJ, FVB/NJ, AKR/J, A/J, DBA/1J, BALB/cJ. The RAB38 protein demonstrates highly conserved amino acid identity between phyla: human/rat (96.2%), human/mouse (93.8%), rat/mouse (95.2%) (*See,* Figure 4, Panel B). The G19V *cht* mutation is located within the highly conserved phosphate/Mg^{2+} (PM) domain and is predicted to directly contact GTP in the nucleotide binding pocket (*See,* Figure 5, Panels A and B).

$Rab38^{cht}$ Results in a Decrease in the Efficiency of Targeting Tyrp1 to End Stage Melanosomes

Analysis of melanocytes cultured from newborn mice revealed that C57Bl/6J $Rab38^{cht}/Rab38^{cht}$ melanocytes contain, small, circular melanosomes with a brown hue similar to those observed in C57Bl/6J $Tyrp1^{b}/Tyrp1^{b}$ melanocytes (*See,* Figure 6, Panel C; and Hearing *et al.,* J Ultrastruct Res 43:88-106 [1973]), but distinct from the intensely black, oval melanosomes seen in C57Bl/6J +/+ melanocyte cultures (*See,* Figure 6, Panels A and B). Given that *Tyrp1* mutations cause the switch from black to brown pigment in *brown* mice, and given that Rab GTPases play a central role in protein trafficking (Schimmoller *et al.,* J Biol Chem 273:22161-22164 [1998]; and Chavrier and Goud, Curr Opin Cell Biol 11:466-475 [1999]), the targeting of TYRP1 to the melanosome was contemplated to be defective in $Rab38^{cht}/Rab38^{cht}$ melanocytes. Consistent with this, endstage melanosomes in the $Rab38^{cht}/Rab38^{cht}$ melanocytes stain much more weakly for TYRP1 than do end stage melanosomes in the control melanocytes (*See,* Figure 7, Panels A-D). Additionally, GFP-tagged RAB38 co-localizes with end stage melanosomes in wild type cells (*See,* Figure 7, Panels E and F). Thus, RAB38 is contemplated to regulate traffic of vesicular intermediates that move Tyrp1 from the trans-golgi network (TGN) to end stage melanosomes.

$Rab38^{cht}$ Does Not Result in Platelet Storage Defects

Subsets of mouse coat color mutants with mutations in genes involved in vesicular trafficking, such as *pale ear* and *beige*, also cause platelet aggregation defects modeling HPS and CHS respectively. To further analyze the pathology of $Rab38^{cht}/Rab38^{cht}$ mice, bleeding times were measured as an assay of platelet function. No difference was observed between wildtype and $Rab38^{cht}/Rab38^{cht}$ mice (2.53 vs. 2.41 minutes; p= 0.7). This observation is consistent with $Rab38^{cht}/Rab38^{cht}$ being a genocopy for OCAIII ($Tyrp1^{b}$) mouse model, but not for either HPS or CHS. Taken together, the data presented herein indicate that RAB38 is required for the efficient

targeting of TYRP1 to pigmented melanosomes, and suggests that *Rab38^{cht}/Rab38^{cht}* is a genocopy of the *Tyrp1^{b}/Tyrp1^{b}* OCAIII mouse model.

***Rab38* is a Melanocyte Pigmentation Gene in Mammals**

Thus, by using cDNA microarray expression profiling, *Rab38* has been identified as an important gene involved in melanocyte pigmentation. Hierarchical clustering of expression patterns grouped *Rab38* with nine previously identified melanocyte genes known to function in a melanocyte-specific fashion. These genes include *DCT, TRYP1,* and *PMEL17* which are essential for melanosome function; *MELAN-A/MART1* and *MLSN* which are important melanoma antigens (Chen *et al.,* Proc Natl Acad Sci USA 93:5915-5919 [1996]; and Duncan *et al.,* J Clin Oncol 19:568-576 [2001]); *AIM-1* which has recently been identified as the gene responsible for B in medaka (Fukamachi *et al.,* Nat Genet 28:381-385 [2001]), *underwhite* in mice (Newton *et al.,* Am J Hum Gent 69:981-988 [2001]), and OCA4 in humans (Newton *et al.,* Am J Hum Gent 69:981-988 [2001]); *CHS1* which functions in melanosome/lysosome vesicle trafficking (Introne *et al.,* Mol Genet Metab 68:283-303 [1999]); and *PAX3,* a paired box transcription factor that regulates melanocyte gene expression (Watanabe *et al.,* Nat Genet 18:283-286 [1998]; Potterf *et al.,* Human Genet 107:1-6 [2000]; and Hornyak *et al.,* Mech Dev 101:47-59 [2001]), including expression of *TYRP1* (Galibert *et al.,* J Biol Chem 274:26894-26900 [1999]). Mutations in seven of these genes have been identified in human and/or murine disorders associated with variations in pigmentation (*See,* Figure 1).

Rab38 was assessed as a candidate gene for the *cht* locus for three reasons. First, comparative genomic analysis predicted a co-localization of the human *Rab38* gene to the region of the *cht* locus in the mouse genome. Second, the expression of *Rab38* was found to be restricted to those cell types affected in *cht/cht* mice (*See,* Figure 2, and Jager *et al.,* Cancer Res 60:3584-3591 [2000]). Finally, *Rab38* is a member of a family of proteins that are known to play a crucial role in vesicular

trafficking (Nielsen *et al.*, Nat Cell Biol 1:376-382 [1999]; and Scott and Zhao, J Invest Dermatol 116:296-304 [2001]).

Sequence analysis of the *Rab38* coding region from *cht* mice revealed a G146T transversion in exon 1. This sequence alteration is likely to be the causative mutation, since this allele arose as a spontaneous mutation on a C57Bl/6J background. Moreover, the G146T alteration results in a Gly to Val substitution within the GTP binding pocket of RAB38. Crystal structure analysis of RAB3A, which is used as a model for other Rab proteins, predicts that this amino acid residue directly contacts the GTP within the nucleotide binding pocket (Dumas *et al.*, Structure Fold Des 7:413-423 [1999]). Furthermore, a mutation of the analogous amino acid residue in RAB5, a Rab that regulates the homotypic fusion of endosomes, results in an increased rate of GDP dissociation *in vitro*, and the stimulation of endosome fusion *in vivo* (Li and Liang, Biochem J 355:681-689 [2001]). Additional support for the functional relevance of this mutation comes from studies of the Ras protein. Substitutions in Ras at the analogous G13 residue, including the same G to V mutation as in *Rab38^{cht}*, have been identified in acute myeloid leukemia (Bos *et al.*, Nature 315:726-730 [1985]; and Stirewalt *et al.*, Blood 97:3589-3595 [2001]). Based upon the analyses disclosed herein and on these observations, the Gly to Val mutation in RAB38 is contemplated to disrupt RAB38 function *in vivo*.

The coat color of *Rab38^{cht}/Rab38^{cht}* mice closely resembles that of the *brown (Tyrp1^{b}/Tyrp1^{b})*, OCAIII mouse model. The *brown* mouse model contains a defect in a melanin biosynthesis gene *Tyrp1*, resulting in a coat color change of the C57BL/6J mouse from black to brown. TYRP is a melanosomal membrane glycoprotein, which functions both as a DHICA oxidase enzyme and to provide structural stability to TYR in the melanogenic enzyme complex. TRYP1 is believed to transit from the trans-golgi network (TGN) to stage II melanosomes via clatherin coated vesicles, possibly by first passing through an uncharacterized sorting compartment (Marks and Seabra, Nat Rev Mol Cell Biol 2:738-748 [2001]). Based upon the similar coat phenotype and predicted Rab protein function, RAB38 is contemplated to be

specifically involved in trafficking of melanosomal proteins like TYRP1, to the melanosome. Consistent with this, GFP-tagged RAB38 co-localizes with melanosomes in pigmented melanocyte lines in culture and TYRP1 is inefficiently targeted to pigmented end stage melanosomes in *Rab38^{cht}/Rab38^{cht}* melanocytes. The brown coat color observed in *Rab38^{cht}/Rab38^{cht}* mice is contemplated to be the result of a reduced amount of melanosomal TYRP1. Thus, RAB38 is implicated in the vesicle trafficking required for proper targeting of proteins, such as TYRP1, to melanosomes.

The formation of melanosomes and melanin pigment deposition within them requires a series of specific vesicular trafficking steps (King *et al.*, in Scriver *et al.* (eds.) The Metabolic Basis of Inherited Disease, 7th ed. (McGraw-Hill, New York) pp.4353-4392 [1995]; and Marks and Seabra, Nat Rev Mol Cell Biol 2:738-748 [2001]). Comparison of the phenotype of *Rab38^{cht}* mice to other mouse mutants where defects in the trafficking of proteins has been identified is contemplated to provide insight into the site of action of RAB38. Four genes involved in HPS (*HPS1*, *AP3* and *HPS3* and *HPS4)*, when mutated result in the mouse models *pale ear (ep)*, *mocha*, *cocoa*, and *light ear*, respectively. For each of these mouse models the color of melanin produced by the melanosome is lighter in color or of a brown hue. Interestingly, similar to what is seen in *Rab38^{cht}/Rab38^{cht}* derived melanocytes, melanocytes from *Hps1^{ep}/Hps1^{ep}* mutants also exhibit a mislocalization of TYRP1 into membraneous complexes rather than pre-melanosomes (Sarangarajan *et al.*, J Invest Dermatol 117:641-646 [2001]), again yielding a brown mouse. In addition to melanosome pigment defects, HPS mice also exhibit enlargement of melanosomes and lysosomes, and reduced platelet cell aggregation (Swank *et al.*, Pigment Cell Res 13:59-67 [2000]; and Introne *et al.*, Mol Genet Metab 68:282-303 [1999]). This suggests the involvement of HPS genes in early vesicle sorting events that affect lysosomes, as well as melanosomes. However, it appears that *Rab38^{cht}* is not in the same class of mutants as those of the HPS mouse models, since *Rab38^{cht}/Rab38^{cht}* mice do not exhibit enlarged melanosomes or lysosomes, or defects in platelet function. Thus, although both HPS1 and RAB38 appear to be involved in the proper sorting of

TYRP1, this regulation appears to be occurring at different steps in the trafficking process. Since RAB38 appears to affect melanosome trafficking only, RAB38 is contemplated to be involved in vesicle trafficking downstream of the HPS genes.

Rab38^{cht} mice appear to be a genocopy of the TYRP1^{b}, OCA mouse model, due to the essential role of RAB38 in proper TYRP1 trafficking to late stage melanosomes, thus mimicking the cellular and clinical phenotype. OCA is a heterogeneous genetic disorder that has been associated with mutations in *TYR* (OCAI), *P* (OCAII) *TYRP1* (OCAIII) and *AIM1* (OCAIV). However, approximately 10% of patients clinically diagnosed with OCA do not have mutations in any of these genes. Given the heterogeneity of OCA and the predicted role of RAB38 in TYRP1 sorting, *Rab38* is contemplated to be a candidate gene for patients with OCA, particularly when a molecular defect in *TYR, P, TYRP1* or *AIM1* has not been found.

Definitions

To facilitate understanding of the invention, a number of terms are defined and discussed below.

The terms "nucleic acid," "nucleic acid sequence," and "nucleotide sequence," as used herein refer to an oligonucleotide or polynucleotide, and fragments or portions thereof, and to DNA or RNA of genomic or synthetic origin which can be single- or double-stranded, and represent the sense or antisense strand.

As used herein, the terms "restriction endonuclease" and "restriction enzyme" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence, referred to as a "restriction site."

As used herein, the terms "complementary" or "complementarity" are used in reference to antiparallel polynucleotides (*i.e.,* a sequence of nucleotides) related by the base-pairing rules. For example, the sequence 5'-AGTTC-3' is complementary to the sequence 3'-TCAAG-5'.

"Amplification" is defined as the production of additional copies of a nucleic acid sequence and is generally carried out using polymerase chain reaction (PCR) or

other technologies well known in the art (*e.g.*, Dieffenbach and Dveksler, *PCR Primer, a Laboratory Manual*, Cold Spring Harbor Press, Plainview NY [1995]). As used herein, the term "polymerase chain reaction" ("PCR") refers to the Mullis method for increasing the concentration of a segment of a target sequence in a mixture of genomic DNA without cloning or purification (*See e.g.*, U.S. Patent Nos. 4,683,195, 4,683,202 and 4,965,188, hereby incorporated by reference). This process for amplifying the target sequence consists of introducing a large excess of two oligonucleotide primers to the DNA mixture containing the desired target sequence, followed by a precise sequence of thermal cycling in the presence of a DNA polymerase. The two primers are complementary to their respective strands of the double stranded target sequence. To effect amplification, the mixture is denatured and the primers then annealed to their complementary sequences within the target molecule. Following annealing, the primers are extended with a polymerase so as to form a new pair of complementary strands. The steps of denaturation, primer annealing and polymerase extension (DNA synthesis) are typically reiterated many times (*i.e.*, denaturation, annealing and extension constitute one "cycle"; there usually are numerous "cycles") to obtain a high concentration of an amplified segment of the desired target sequence. The length of the amplified segment of the desired target sequence is determined by the relative positions of the primers with respect to each other, and therefore, this length is a controllable parameter. By virtue of the repeating aspect of the process, the method is referred to as the "polymerase chain reaction" (hereinafter "PCR"). Because the desired amplified segments of the target sequence become the predominant sequences (in terms of concentration) in the mixture, they are said to be "PCR amplified."

As used herein, the terms "PCR product" and "amplification product" refer to the resultant mixture of compounds after two or more cycles of the PCR steps of denaturation, annealing and extension are complete.

The terms "peptide," "polypeptide" and "protein" all refer to a primary sequence of amino acids that are joined by covalent peptide linkages. In general, a peptide consists of a few amino acids, typically from 2-25 amino acids, and is shorter than a protein. "Polypeptides" encompass both peptides and proteins.

As used herein the term "portion" when in reference to a gene refers to fragments of that gene. In some embodiments, the fragments range in size from ten nucleic acids to the entire nucleic acid sequence minus one nucleic acid.

As used herein, the term "purify" or "purifying" refers to the removal of at least one contaminant from a sample. As used herein, the term "substantially purified" refers to molecules, either nucleic acids or amino acid sequences, that are removed from their natural environment, "isolated" or "separated," and are largely free from other components.

The term "gene" refers to a nucleic acid (*e.g.*, DNA) sequence comprised of parts, that when appropriately combined in either a native or recombinant manner, provide some product or function. In addition to the coding region of the nucleic acid, the term "gene" also encompasses the transcribed nucleotide sequences of the full-length mRNA adjacent to the 5' and 3' ends of the coding region. These noncoding regions are referred to as 5' and 3' untranslated sequences (5' UT and 3' UT). Both the 5' and 3' UT may serve regulatory roles, including translation initiation, post-transcriptional cleavage and polyadenylation. In preferred embodiments, a mammalian "*Rab38* gene" is provided.

In some embodiments, the "genomic" form of a gene contains the sequences of the transcribed mRNA, as well as other non-coding sequences. "Introns" or "intervening sequences" are segments of a gene which are contained in the primary transcript (*i.e.*, hetero-nuclear RNA, or hnRNA), but are spliced out to yield the processed mRNA form. Conversely, "exons" are the segments of a gene corresponding to the processed mRNA sequence.

The terms "in operable combination," and "operably linked" when used in reference to nucleic acid herein are used to refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. In preferred embodiments of the present invention, a mammalian *Rab38* gene in "operable combination" with a promoter and/or an enhancer is provided.

As used herein, the term "vector" is used in reference to nucleic acid molecules that transfer DNA segment(s) from one cell to another. In some embodiments, a vector "backbone" comprises those parts of the vector which mediate its maintenance and enable its intended use (*e.g.*, sequences necessary for replication, genes imparting drug or antibiotic resistance, a multiple cloning site, and possibly operably linked promoter/enhancer elements which enable the expression of a cloned nucleic acid). Vectors are often derived from plasmids, bacteriophages, or plant or animal viruses.

As used herein, the term "wild type" refers to a gene or gene product which has the characteristics of that gene or gene product when isolated from a naturally occurring source. A wild-type gene is that which is most frequently observed in a population and is thus arbitrarily designated the "normal" or "wild-type" form of the gene.

In contrast, the terms "mutant" and "mutation" refer to a gene or gene product which displays modifications in sequence and or functional properties (*i.e.*, altered characteristics) when compared to the wild-type gene or gene product. It is noted that naturally-occurring mutants can be isolated and these are identified by the fact that they have altered characteristics when compared to the wild-type gene or gene product. In some embodiments, the present invention provides a mutant *Rab38* gene or RAB38 protein. In preferred embodiments, the *chocolate Rab38*/RAB38 mutant is provided.

The term "candidate agent" refers to any molecule of any composition, including proteins, peptides, nucleic acids, lipids, carbohydrates, organic molecules, inorganic molecules, and/or combinations of molecules which are suspected to be capable of producing a physiological or biological response.

As used herein, the term "modulate" refers to a change in the activity of RAB38. For example, modulation may cause an increase or a decrease in enzymatic activity, binding characteristics, or any other biological, or functional properties of RAB38.

The term "melanocyte" as used herein, refers to special cells in the skin and the eye that synthesize melanin pigments. Clusters of melanocytes often appear on the

skin as moles. The term "melanosome" refers to the melanin-producing organelle of melanocytes.

As used herein, the terms "GTPase activity" and "guanosine triphosphatase activity" refer to the enzyme activity that hydrolyses GTP to produce GDP and orthophosphate. GTPase activity is regulated by GTPase activating proteins (activation) and by guanine nucleotide releasing proteins (inhibition). In the context of the invention, GTPase activity refers to "RAB38 activity" or the GTPase activity of RAB38. RAB38 is inactive when bound to GDP, and active when bound to GTP. Thus, the term "GTP binding" refers to the binding of GTP by a GTPase (*e.g.*, RAB38), while the term "GDP release" refers to the release of GDP by a GTPase (*e.g.*, RAB38). GTPases of the RAB family have been implicated in the process of vesicle trafficking. In the context of the invention, the term "RAB activity" encompasses "RAB38 trafficking" or the transport of RAB38 to melanosomes of melanocytes, and "TYRP1 trafficking" or the transport of TYRP1 to melanosomes.

EXPERIMENTAL

The following examples are provided in order to demonstrate and further illustrate certain preferred embodiments and aspects of the present invention and are not to be construed as limiting the scope thereof.

In the experimental disclosure which follows, the following abbreviations apply: °C (degrees Centigrade); BSA (bovine serum albumin); DMEM (Dulbecco's modified eagle's medium); FBS (fetal bovine serum); H_2O (water); aa (amino acid); bp (base pair); kb (kilobase pair); EST (expressed sequence tag); kD (kilodaltons); gm (grams); µg (micrograms); mg (milligrams); ng (nanograms); µl (microliters); ml (milliliters); mm (millimeters); nm (nanometers); µm (micrometer); M (molar); mM (millimolar); µM (micromolar); U (units); MW (molecular weight); sec (seconds); min(s) (minute/minutes); hr(s) (hour/hours); CO_2 (carbon dioxide); Cy3 (indocarbocyanine); Cy5 (indodicarbocyanine); (DEPC (diethyl pyrocarbonate); dNTPs (deoxynucleotides); $MgCl_2$ (magnesium chloride); PBS (phosphate-buffered saline [150 mM NaCl, 10 mM sodium phosphate buffer, pH 7.2]); SDS (sodium dodecyl sulfate);

SSC (saline sodium citrate buffer); TBS (Tris-buffered saline); PCR (polymerase chain reaction); RT (reverse transcription); w/v (weight to volume); v/v (volume to volume); Applied Biosystems (Applied Biosystems, Foster City, CA); Axon (Axon Instruments Inc., Foster City, CA); GeneCodes (GeneCodes, Ann Arbor, MI); Invitrogen (Invitrogen Corp., Carlsbad, CA); Jackson (Jackson Laboratory, Bar Harbor, ME); NEB (New England Biolabs, Inc., Beverly, MA); NIH (National Institutes of Health, Bethesda, MD); Qiagen (Qiagen, Valencia, CA); Roche (Roche, MannHeim, Germany); and Signet (Signet Laboratories, Dedham, MA).

EXAMPLE 1

Cell Culture

All cells for microarray analysis were grown to 90% confluence at 37°C in 5% CO_2. The cell lines used in these experiments were obtained from Jeffry Trent (NIH). Melanoma cell lines were grown in RPMI media containing 10% FBS, 2 mM L-glutamine and 100 units/ml each of penicillin and streptomycin. Media for MnSOD6 cl1 also contained 500 μg/ml geneticin. 293T, U138 and HeLa cells were grown in DMEM media containing 10% FBS, 2mM L-glutamine and 100 units/ml each of penicillin and streptomycin. Primary murine melanocytes were cultured as previously described (Wu *et al.,* J Cell Sci 114:1091-1100 [2001]. Melan-a cells were cultured in RPMI 1640 media containing 10% FBS, 2 mM L-glutamine, 10 mM sodium pyruvate, 100 units/ml each of penicillin and streptomycin, 200 nm 12-*O*-tetradecanoylphorbol 13-acetate, 0.01 mM sodium bicarbonate and 0.1 mM 2-mercaptoethanol in 5% CO_2.

EXAMPLE 2

RNA Preparation

Cells for microarray analysis were obtained in pools of four 500 cm^2 dishes, that were harvested by scraping, washed in PBS, and pelleted. Pellets were lysed in 10 ml Trizol reagent (Invitrogen). Two ml of chloroform were added, the sample was shaken, and then centrifuged to separate phases. The aqueous layer was removed and

an equal volume of 75% ethanol was added dropwise while vortexing. Sample was applied to a RNeasy maxi column (Qiagen) and the manufacturer's purification protocol was followed. Samples were eluted in water, precipitated with 3 M sodium acetate and stored at -80°C. RNA pellets were resuspended in DEPC water to 1 μg/μl concentration, and applied to a Microcon 100 column. RNA samples were centrifuged and concentrated to 7-10 μg/ul.

EXAMPLE 3

Labeling and Hybridization

RNA was reverse transcribed to fluorescent labeled cDNA and co-hybridized on slides in experimental/reference pairs. Expressed sequence tag (EST) clone inserts were prepared and applied to slides as described (DeRisi *et al.,* Nat Genet 14:457-460 [1996]). Reversed transcribed (RT) fluorochrome labeled cDNA was generated as known in the art (*See,* The National Human Genome Research Initiative's Microarray Project web site). For reactions, 60 μg of total RNA (Cy3) or 120 μg of total RNA (Cy5) were used. Hybridizations were carried out in a final volume of 40 μl at 65°C in a humidified chamber for 16 hr. Slides were washed at room temperature in 0.5X SSC/0.1% SDS for 3 min followed by a second wash in 0.6X SSC for 3 min. Slides were immediately spun dry by centrifugation.

EXAMPLE 4

Image Acquisition and Analysis

Fluorescence signal intensities for Cy3 (532 λ) and Cy5 (635 λ) fluorochromes were obtained using a Genepix 4000a scanner (Axon) at 10 μM resolution. A set of 88 housekeeping control genes was used to normalize for labeling efficiency (Loftus *et al.,* Proc Natl Acad Sci USA 96:9277-9280 [1999]). Expression profile analysis was performed with a clustering algorithm using average-linkage method and Pearson's correlation similarity measurement (*See,* The National Human Genome Research Initiative's Genome Clustering web site).

EXAMPLE 5

Organization of the Mouse *Rab38* Gene

To facilitate mutation screening of the mouse *Rab38* gene, database searches were employed to identify genomic DNA adjacent to *Rab38* coding sequences. *Rab38* mRNA sequence (SEQ ID NO:9 and GenBank Accession No. AY062237), was BLASTed against mouse genomic sequencing trace archives (*See,* The National Center for Biotechnology Information's web site for Trace Archive Querying). Significant similarities were determined by a returned BLAST score of greater than 200. Relevant data were downloaded and aligned to the mRNA sequence using Sequencher version 3.1.1 (GeneCodes). Genomic organization was confirmed by using the Spidey program (*See,* The National Center for Biotechnology Information's web site for Spidey). Gene organization was also experimentally confirmed through PCR and DNA sequencing of genomic fragments. This analysis revealed that *Rab38* is composed of three exons: exon 1, nucleotides 1-292; exon 2, nucleotides 292-572; and exon 3, nucleotides 573-1439 (nucleotide positions refer to GenBank Accession No. AY062237). The confirmed exon and surrounding intron sequences have been deposited into GenBank as: exon 1, AF448441 (SEQ ID NO:10); exon 2, AF448442 (SEQ ID NO:11); and exon 3, AF448443 (SEQ ID NO:12).

EXAMPLE 6

In Situ Hybridization

Timed matings were used to obtain staged FVB/NJ (Jackson) mouse embryos, and E0.5 was designated as noon on the day of vaginal plug formation. Embryos were fixed overnight in 4% paraformaldehyde in PBS. Digoxigenin conjugated probes were synthesized by reverse transcription (RT) of linearized plasmids and/or PCR products with RT binding site linkers (Roche). The following DNA sources were used for probe synthesis: Tyrosinase, cDNA clone 4633402C07; *Tyrp1*, RT-PCR from B16 cell line total RNA (TYRP15'T3F 5'-GCGCGAATTA ACCCTCACTA AAGGGTCTGA GCACCCCTGT CTTCT-3', SEQ ID NO:13; TYRP15'T7R 5'-GCGCGTAATA

CGACTCACTA TAGGGCCCAG TTGCAAAATT CCAGT-3', SEQ ID NO:14); *Dct*, cDNA ; *Aim1/Matp*, RIKEN cDNA clone G370045L22; *Mlsn1* RT-PCR from B16 cell line total RNA (MLSN R T7 5'-GCGGGTAATA CGACTCACTA TAGGGGCCAC AAACATGTCC TACTTAC-3', SEQ ID NO:15; MLSN FT3 5'-GCGCGAATTA ACCCTCACTA AAGGGAAGCT TCCGGACTCT CTAC-3', SEQ ID NO:16); *Rab38*, Riken cDNA clone 23-10011-F14. *In situ* hybridizations were performed using published protocols (Wilkinson and Nieto, Methods Enzymol 225:361-373 [1993]), with the following modifications. After probe hybridization, Ribonuclease A digestion was omitted, TBS was used in place of PBS, and the substrate BM-purple (Roche) was used in place of 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium.

EXAMPLE 7

Mutation Detection

The mouse *Rab38* gene homologue was screened for mutations by PCR amplification and DNA sequencing of exon-containing genomic segments. The following primer pairs were designed to amplify the three protein coding exons, as well as a small amount of flanking intron DNA: *Rab38* Ex1F 5'-TAGGAAGGAG GATTAAACCC G-3' (SEQ ID NO:17) and *Rab38* Ex1R 5'-GAACTCCTCA TGGCTCACTC C-3' (SEQ ID NO:18) yielding a 428 bp product; *Rab38* Ex2F 5'-GGATATGAAG CTCCAGTGTA GTGTAC-3' (SEQ ID NO:19) and *Rab38* Ex2R 5'-CACTGGACAG AAACATTATT GTCAC-3' (SEQ ID NO:20) yielding a 464 bp product; and *Rab38* Ex3F 5'-AAGTTATCAG CCAGTGAGAT ACTGTG-3' (SEQ ID NO:21) and *Rab38* Ex3R 5'-CACATGTGGT ATATCTATCC TGACG-3' (SEQ ID NO:22) yielding a 526 bp product. PCR reactions contained 1.5 μM of each primer, 0.2 μM dNTPs, 1.5 μM $MgCl_2$, 1 unit AmpliTaq DNA Polymerase (Applied Biosystems), 1X of the manufacturer's 10X buffer, and 40 ng of mouse genomic DNA. Thermal cycling consisted of an initial denaturation for 2 min at 93°C, followed by 40 cycles of 93°C for 10 sec, 55°C for 5 sec, and 72°C for 30 sec. A final extension at 72°C was performed for 7 min. Following separation on a 1%

agarose gel, PCR products were excised and purified using the QIAquick Gel Extraction Kit (Qiagen). DNA sequencing was performed with BigDye terminator chemistry, and a model 3100 DNA sequencing instrument (Applied Biosystems). The cycle-sequencing routine was 30 cycles of 92°C for 20 sec, 55°C for 10 sec, and 60°C for 4 min with a 20 µL reaction containing 8 µL of BigDye cocktail, 0.5 µL of a 25 µM primer solution, 6.5 µL of water, and 5 µL of PCR product (at ~50 ng/µL). Data was extracted and analyzed using Sequencing Analysis version 3.3 (Applied Biosystems) and aligned with Sequencher software version 3.1.1 (GeneCodes). Alignments included sequence data derived from wildtype C57Bl/6J and heterozygous *cht*/+ mice, as well as mouse *Rab38* mRNA sequence (GenBank Accession No. AY062237).

EXAMPLE 8

Mutation Confirmation and Genotyping

Primers were designed to amplify a 213 bp fragment surrounding the G146T sequence change (cht Ex1F 5'-GGCCTCCAGG ATGCAGACAC C-3', SEQ ID NO:23; cht Ex1R 5'-CCAGCAATGT CCCAGAGCTG C-3', SEQ ID NO:24). PCR amplification was performed as described in Example 7. *Sex*AI and *Bsa*JI restriction digests were performed using 20 µL restriction enzyme digests containing 10 µL of PCR product, 2.5 units enzyme (NEB) along with 1X of the supplied BSA and digest buffer. Reactions were incubated overnight at the manufacturer's suggested temperature and electrophoresed on a 2% agarose gel to visualize band patterns.

EXAMPLE 9

Cell Transfection

GFP-RAB38 constructs were generated by PCR amplifying mouse *Rab38* with *att* site linker primers (AttB1-RRab 5'-GGGGACAAGT TTGTACAAAA AAGCAGGCTC CATGCAGACA CCTCACAAG-3', SEQ ID NO:25 and AttB2-RRab-STP 5'-GGGGACCACT TTGTACAAGA AAGCTGGGTT CTAGGATTTG GCACAGCCAG A-3', SEQ ID NO:26) and "Gateway " cloning into

pDest 53 (Invitrogen) as per manufacturer's instructions. GFP-RAB38 was transfected into melan-a cells using LipofectAMINE 2000 (Invitrogen) with a DNA / LipofectAMINE 2000 ratio of 1.6 g/4 µl in a 4 cm^2 surface area as per manufacturer's instructions. After 72 hours, cells were fixed and stained with a 1:200 dilution of the TYRP1-reactive antibody MEL5 (Signet) as previously described (Wu *et al.*, J Cell Sci 110:847-859 [1997]).

EXAMPLE 10

Bleeding Times

Bleeding times were assayed in four C57Bl/6J *Rab38^{cht}/Rab38^{cht}* and four C57Bl/6J animals, as described in the art (Sviderskaya *et al.*, Genetics 148:381-390 [1998]). Assayed mice were 6–12 weeks of age. A 2 mm portion of the tail was removed and the cut tail immediately immersed in saline at 37°C. Each mouse was maintained in a horizontal position in a restrainer with the tip of the tail held 4–5 cm below the body. Bleeding time was that required for the small stream of blood to stop abruptly.

All publications and patents mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described method and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in the art and/or related fields are intended to be within the scope of the present invention.

CLAIMS

What is claimed is:

1. An isolated nucleic acid comprising a sequence selected from the group consisting of SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11, and SEQ ID NO:12.

2. The isolated nucleic acid of Claim 1, wherein said nucleic acid is deoxyribonucleic acid.

3. An isolated nucleic acid, wherein said nucleic acid is the complement of the nucleic acid of Claim 1.

4. A vector comprising the nucleic acid of Claim 1.

5. A host cell comprising the vector of Claim 4.

6. An isolated protein encoded by the nucleic acid sequence set forth in SEQ ID NO:9.

7. A method for detecting mutations in *Rab38* comprising the steps of:

a) amplifying at least a portion of *Rab38* from genomic DNA to yield a *Rab38* amplification product;

b) purifying said *Rab38* amplification product; and

c) sequencing said *Rab38* amplification product.

8. The method of Claim 7, wherein said amplifying is accomplished using a polymerase chain reaction.

9. The method of Claim 7, wherein said at least a portion of *Rab38* is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence.

10. The method of Claim 9, wherein said at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3.

11. The method of Claim 7, wherein said genomic DNA is mammalian genomic DNA.

12. The method of Claim 7, wherein said purifying is accomplished using size selection.

13. A method for detecting mutations in *Rab38* comprising the steps of:

a) amplifying at least a portion of *Rab38* from genomic DNA to yield a *Rab38* amplification product;

b) digesting said *Rab38* amplification product to yield a digested *Rab38* amplification product; and

c) electrophoresing said digested *Rab38* amplification product.

14. The method of Claim 13, wherein said amplifying is accomplished using a polymerase chain reaction.

15. The method of Claim 13, wherein said at least a portion of *Rab38* is selected from the group consisting of at least one *Rab38* exon, at least one *Rab38* intron, the *Rab38* 5' untranslated sequence, and the *Rab38* 3' untranslated sequence.

16. The method of Claim 15, wherein said at least one *Rab38* exon is selected from the group consisting of *Rab38* exon 1, *Rab38* exon 2, and *Rab38* exon 3.

17. The method of Claim 13, wherein said genomic DNA is mammalian genomic DNA.

18. A method for screening for biologically active agents to modulate RAB38 activity, comprising the steps of:

a) providing:

i) melanocytes comprising RAB38 activity, and

ii) a candidate agent; and

b) exposing said melanocytes to said candidate agent to yield treated melanocytes; and

c) measuring the modulation of said RAB38 activity of said treated melanocytes by said candidate agent.

19. The method of Claim 18, wherein said RAB38 activity comprises GTPase activity.

20. The method of Claim 18, wherein said RAB38 activity comprises GTP binding activity.

21. The method of Claim 18, wherein said RAB38 activity comprises GDP release.

22. The method of Claim 18, wherein said RAB38 activity comprises TYRP1 trafficking to melanosomes.

23. The method of Claim 18, wherein said RAB38 activity comprises RAB38 trafficking to melanosomes.

24. A kit for screening for biologically active agents that modulate RAB38 activity, comprising: a) plurality of melanocytes comprising RAB38 activity, wherein said melanocytes are provided within a container, and b) instructions for determination of RAB38 activity in said melanocytes.

25. The kit of Claim 24, further comprising means to analyze RAB38 activity.

26. The kit of Claim 25, wherein said means to analyze RAB38 activity comprises an assay to assess GTPase activity.

27. The kit of Claim 25, wherein said means to analyze RAB38 activity comprises an assay to assess GTP binding activity.

28. The kit of Claim 25, wherein said means to analyze RAB38 activity comprises an assay to assess GDP release.

29. The kit of Claim 25, wherein said means to analyze RAB38 activity comprises an assay to assess TYRP1 trafficking to melanosomes.

30. The kit of Claim 25, wherein said means to analyze RAB38 activity comprises an assay to assess RAB38 trafficking to melanosomes.

31. A kit for detection of mutations in *RAB38* comprising at least two primer sequences suitable for amplification of at least a portion of *RAB38*, and instructions for utilizing said kit.

32. The kit of Claim 31, wherein said primer sequences are selected from the group consisting of SEQ ID NO:17, SEQ ID NO:18, SEQ ID NO:19, SEQ ID NO:20, SEQ ID NO:21, and SEQ ID NO:22.

33. The kit of Claim 31, wherein said kit is suitable for use in the polymerase chain reaction.

34. The kit of Claim 31, further comprising reagents for digesting nucleic acid.

35. A kit for diagnosing defects in melanosome function, comprising melanocytes comprising *RAB38* and instructions for assessing defects in melanosome function.

36. The kit of Claim 35, further comprising means to analyze RAB38 activity.

37. The kit of Claim 36, wherein said means to analyze RAB38 activity comprises an assay to assess GTPase activity.

38. The kit of Claim 36, wherein said means to analyze RAB38 activity comprises an assay to assess GTP binding activity.

39. The kit of Claim 36, wherein said means to analyze RAB38 activity comprises an assay to assess GDP release.

40. The kit of Claim 36, wherein said means to analyze RAB38 activity comprises an assay to assess TYRP1 trafficking to melanosomes.

41. The kit of Claim 36, wherein said means to analyze RAB38 activity comprises an assay to assess RAB38 trafficking to melanosomes.

ABSTRACT

The present invention relates to compositions and methods involving melanocytes. In particular, the present invention provides compositions and methods involving RAB38 and mutant RAB38, suitable for use in modulation of pigmentation and for use in determining the means to diagnose and/or treat conditions associated with disorders in pigmentation.

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ASSIGNMENT

WHEREAS, WE, William J. Pavan and Stacie K. Loftus, hereinafter referred to as "ASSIGNOR", have invented certain new and useful improvements as described and set forth in the below-identified application for United States Letters Patent:

Title of Invention: **Alteration of RAB38 Function To Modulate Mammalian Pigmentation**

Filing Date: Serial No.:

WHEREAS, the conditions under which said invention was made are such as to entitle the Government under Paragraph 1(a) of Executive order 10096 to the entire right, title, and interest herein, both domestic and foreign; and

WHEREAS, the Government of the United States is desirous of acquiring all domestic and foreign right, title, and interest in the above-mentioned invention described in the provisional application for Letters Patent; and

NOW, THEREFORE, for good and valuable consideration the receipt of which is hereby acknowledged, I hereby assign and transfer to the United States of America, represented by the Secretary, Department of Health and Human Services, the full and exclusive rights in and to said invention in the U.S. and within each and every foreign country in which the Government elects to file and the entire right, title, and interest in and to such application, and any continuations, continuations-in-part, divisions, reissues or extensions thereof, and including priority rights as may be filed in the U.S. and foreign countries, and such Letters Patent as may be granted to be held by the Government to the end of the term for which the same would have been held by the inventors had this assignment not been made.

I further agree to make, execute, and deliver to the Secretary, Department of Health and Human Services, upon request, any and all papers, documents, affidavits, or other instruments that may be necessary in the prosecution of any application or applications for improvements or reissues of Letters Patent, and to assist the Government in every way as may be requested in protecting said invention, provided that any expense of extending such assistance shall be paid by the Government.

IN TESTIMONY WHEREOF, ASSIGNOR has hereunto signed ASSIGNOR's names to this assignment on the date indicated below.

_____ William J. Pavan

_____ Stacie K. Loftus

STATE OF _____)
)
COUNTY OF _____) ss.

On this _____ day of _____, in the year of _____, before me, the undersigned notary public, personally appeared the above-named ASSIGNOR, known to me (or proved to me on the basis of satisfactory evidence) to be the person whose name is subscribed to the within instrument, and acknowledged that he/she executed the same.

_____ NOTARY PUBLIC

SEAL

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	William J. Pavan *et al.*	Group No.:
Serial No.:		Examiner:
Filed:		
Entitled:	**Alteration of RAB38 Function To Modulate Mammalian Pigmentation**	

POWER OF ATTORNEY BY ASSIGNEE AND ASSOCIATE POWER OF ATTORNEY

Assistant Commissioner for Patents
Washington, D.C. 20231

The Government of the United States of America, as represented by the Secretary, Department of Health and Human Services, as Assignee of record of the entire interest of the above-identified application, hereby appoints the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith:

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Jack Spiegel (Reg. No. 34,477)	Steve Ferguson (Reg. No. 38,448)
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Norbert Pontzer (Reg. No. 40,777)	Richard U. Rodriguez (Reg. No. 45,980)
Marlene Shinn (Reg. No. P-46,005)	

all of the Office of Technology Transfer, National Institutes of Health, Rockville, Maryland, with an Associate Power of Attorney to:

Virginia S. Medlen (Reg. No. 32,050)	Maha A. Hamdan (Reg. No. 43,655)
Peter G. Carroll (Reg. No. 32,837)	J. Mitchell Jones (Reg. No. 44,174)
Kamrin T. MacKnight (Reg. No. 38,230)	David J. Wilson (Reg. No. 45,225)
David A. Casimir (Reg. No. 42,395)	Jaen Andrews (Reg. No. 35,051)
Jason R. Bond (Reg. No. 45,439)	Tanya Arenson (Reg. No. 47,391)
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Thomas W. Brown (Reg. No. 50,002)	Thomas C. Howerton (Reg. No. 48,650)

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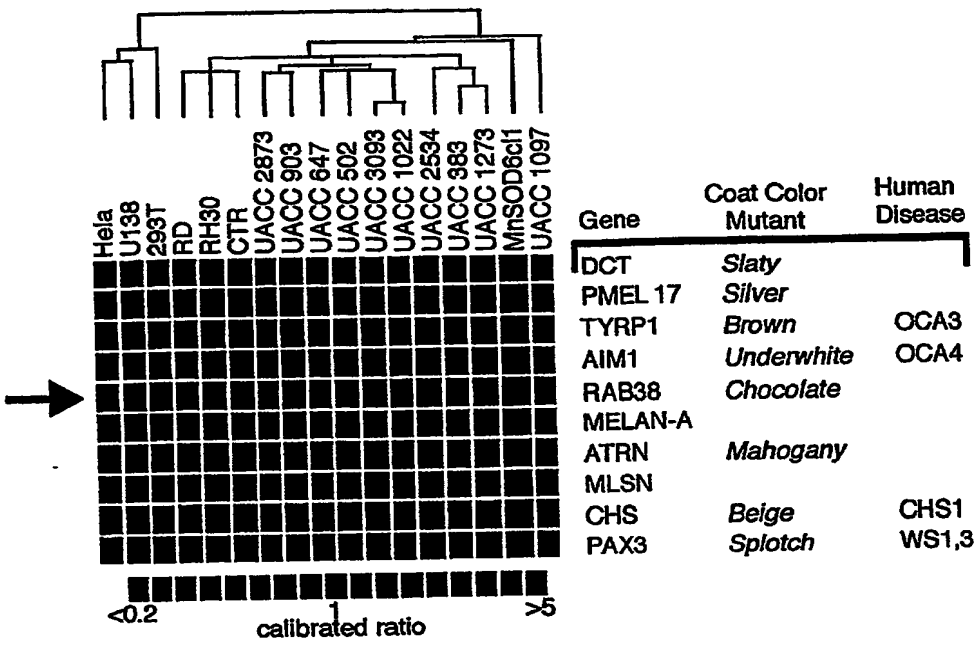
I hereby certify that the Assignment document filed with the above-referenced patent application or filed subsequent to the filing date of the above-referenced patent application, has been reviewed and I hereby certify that, to the best of my knowledge and belief, title is with the Government of the United States.

Dated: _____ By: _____

Name: _____

Title: _____



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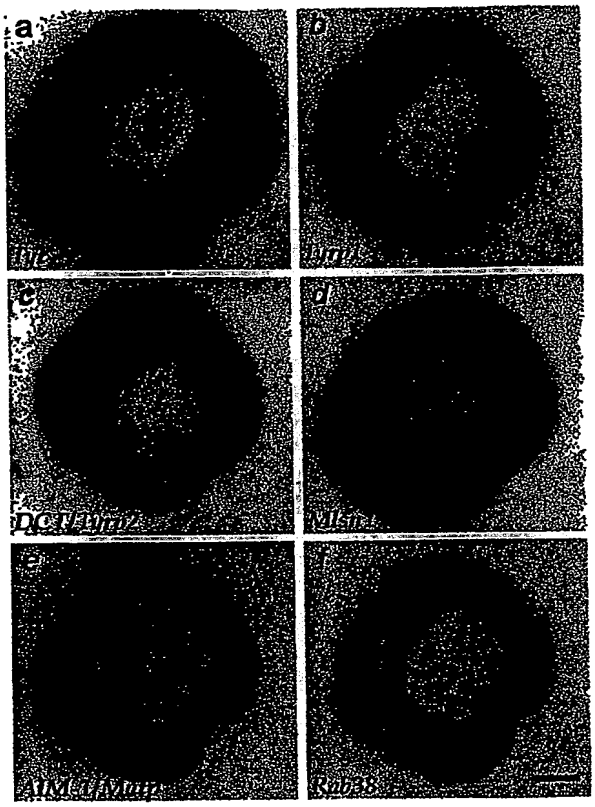

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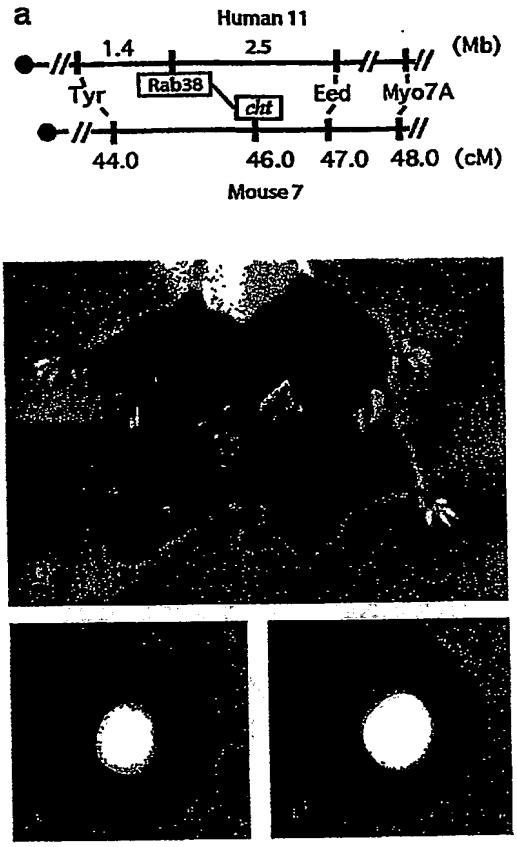

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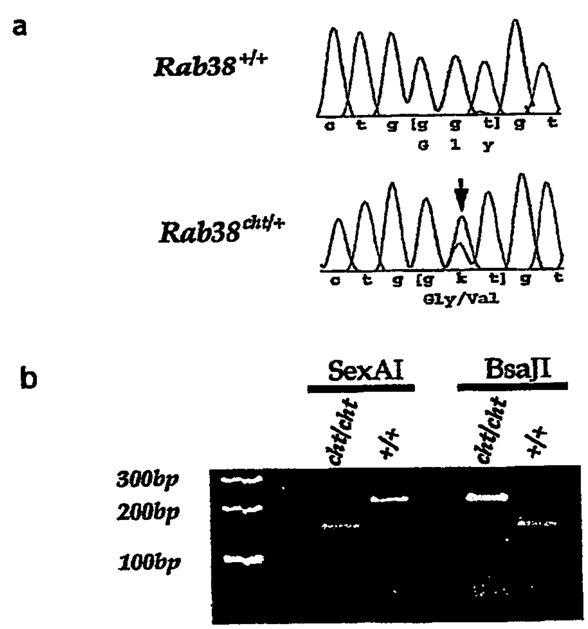

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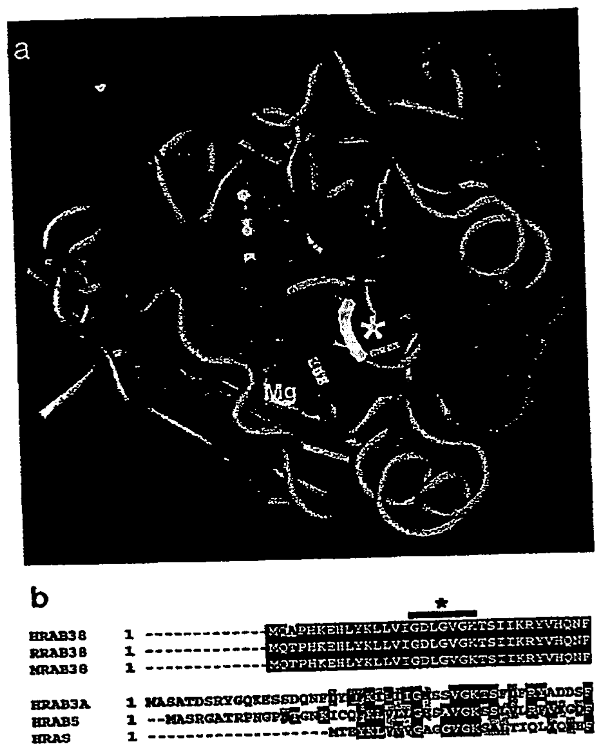
Fig. 4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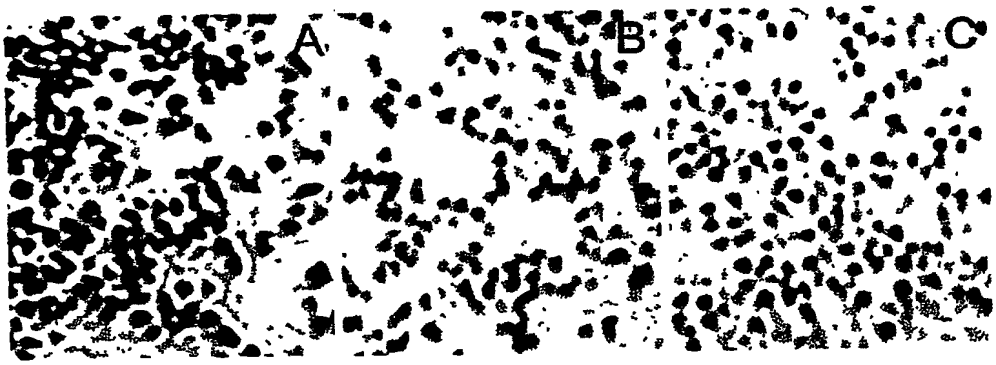

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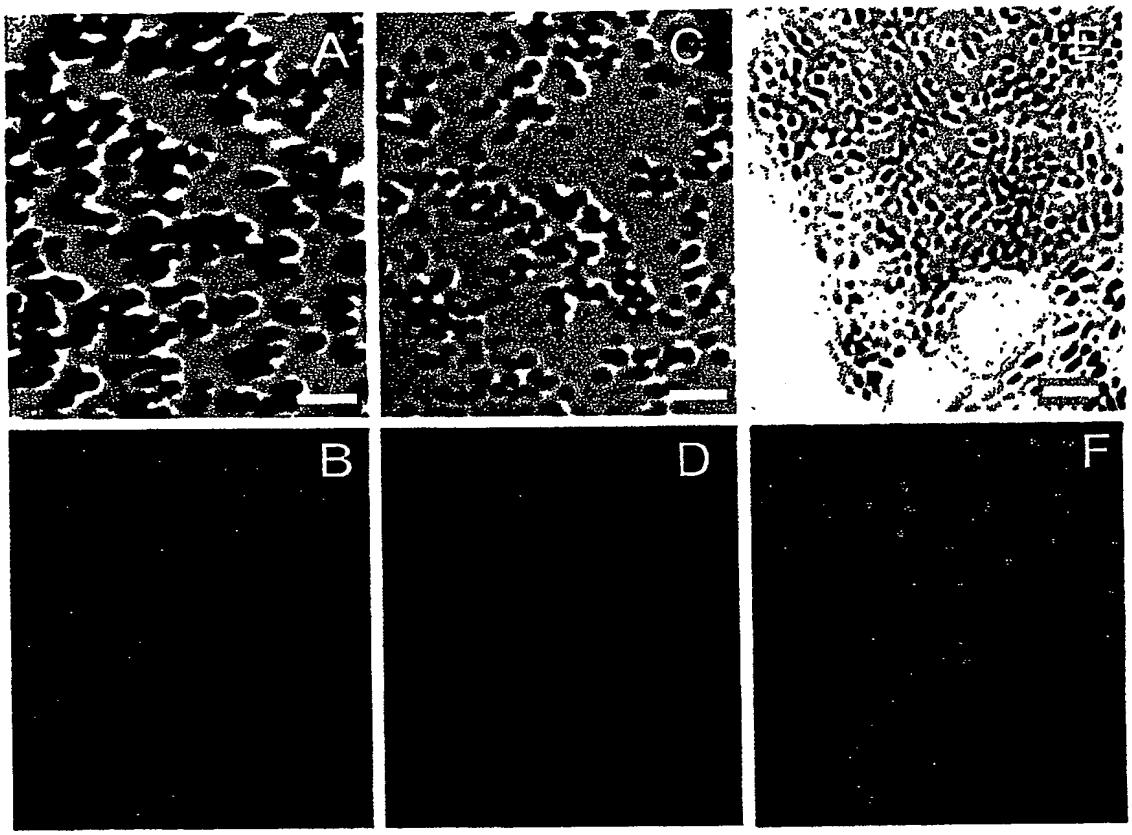

Fig. 7

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Loftus, Stacie K.

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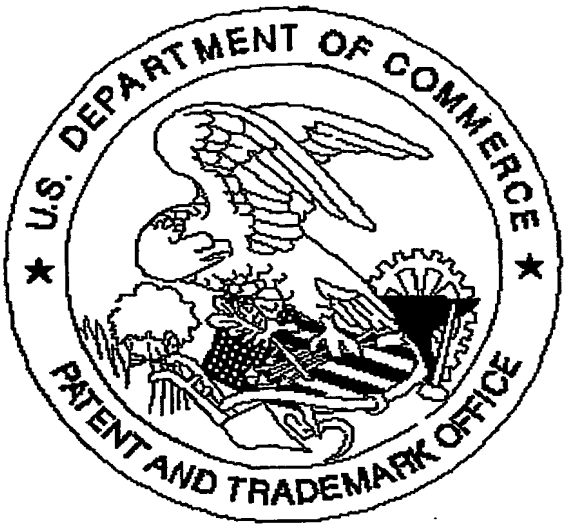

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Pages 26-34 as part of the specification are sequence listing.

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